Journal of the American Association for Laboratory Animal Science
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Vol 63, No 1
January 2024
Pages 49–56

Subcutaneous Alfaxalone-Xylazine-Buprenorphine for Surgical Anesthesia and Echocardiographic Evaluation of Mice (*Mus musculus*)

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Alfaxalone is a commonly used injectable anesthetic in dogs and cats due to its minimal cardiovascular side effects. Data for its use in mice are limited and demonstrate strain- and sex-associated differences in dose-response relationships. We performed a dose-comparison study of alfaxalone-xylazine-buprenorphine (AXB) in Crl:*CFW*(SW) mice. Subcutaneous injection of 50 mg/kg alfaxalone-10 mg/kg xylazine-0.1 mg/kg buprenorphine HCl consistently achieved a surgical plane of anesthesia (loss of toe pinch) for 48.6 ± 4.7 and 60.8 ± 9.6 min in females and males, respectively. The same dose and route of AXB induced a surgical plane of anesthesia in C57Bl/6NCrl (females: 42.3 ± 11.2 min; males: 51.6 ± 12.3 min), NCr-Foxn1^{nu} (females: 76.8 ± 32.5 min; males: 80.0 ± 1.2 min), and NOD.Cg-Prkdc^{SCID}Il2rg^{tm1Wjl}/SzJCr (females: 56.0 ± 37.2 min and males: 61.2 ± 10.2 min) mice. We found no significant difference in the duration of the surgical plane of anesthesia between males and females within the mouse strains Crl:*CFW*(SW), C57Bl/6NCrl, NCr-Foxn1^{nu}, and NOD.Cg-Prkdc^{SCID}Il2rg^{tm1Wjl}/SzJCr. We next performed an echocardiography study (n = 5 per group) of Crl:*CFW*(SW) mice (n = 5 per group) to compare subcutaneous AXB anesthesia with that produced by intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine (KX). AXB induced significantly less bradycardia (295.4 ± 29 bpm) than KX (185.8 ± 38.9 bpm) did, with no significant differences in cardiac output, ejection fraction, end-diastolic volume, end-systolic volume, or fractional shortening. These results suggest that subcutaneous administration of AXB is a viable alternative to KX for inducing a surgical plane of anesthesia in Crl:*CFW*(SW), C57Bl/6NCrl, NCr-Foxn1^{nu}, and NOD.Cg-Prkdc^{SCID}Il2rg^{tm1Wjl}/SzJCr mice, regardless of sex. AXB may also be a better injectable anesthetic option as compared with KX for avoiding adverse cardiac effects in mice.

Abbreviations and Acronyms: AXB, alfaxalone-xylazine-buprenorphine HCl; CO, cardiac output; EF, ejection fraction; FS, fractional shortening; HR, heart rate; KX, ketamine-xylazine; LORR, loss of righting reflex; V;d, end-diastolic volume; V;s, end-systolic volume

DOI: 10.30802/AALAS-JAALAS-23-000090

Introduction

Animals that are used in research often require anesthesia, which can be provided by either inhalation or injection. Inhalant anesthetics are commonly used for both brief and prolonged experimental procedures due to their properties of rapid induction, rapid recovery, and broad safety profile.[5,9,29,33] Disadvantages of inhalant anesthetics include equipment cost, quality assurance to guarantee accurate and consistent exposure of the anesthetic, waste-gas exposure risk to personnel, difficulty in sanitizing equipment, and anatomic incompatibility for procedures involving the face, upper respiratory tract, and oral cavity.

Injectable anesthetics can be used to avoid these disadvantages. A combination of ketamine-xylazine (KX) injected IP has become the most common and easiest method to induce anesthesia in research mice.[3,29] The major disadvantage of the KX combination is an unpredictable plane of anesthesia that ranges from inadequate anesthesia to death.[3,5,15,16] Ketamine also significantly reduces heart rate[4,17,27,28] and therefore may not be suitable for some studies.

Alfaxalone is an injectable neuroactive steroid anesthetic that potentiates GABA type A receptors in the central nervous system, resulting in anesthesia and muscle relaxation.[1,5,6,14,29] A formulation of alfaxalone was launched in 1971 but later was withdrawn from the market because the lyophilized powder and reconstituted cyclodextrin caused histamine release and anaphylactoid reactions.[5,13,29] Since its reformulation in 2014, it has gained popularity because of its ability to provide reliable anesthesia with little to no cardiovascular side effects in dogs and cats.[5,13,18,19,22,23,29] Alfaxalone can be administered by different routes (IP, SQ, IM, and IV), allowing flexibility in experimental use.[22,23,33]

A few studies of alfaxalone in mice have shown that different anesthetic effects are achieved depending on the route of administration, sex, and strain.[5,7,34] A previous study found that a combination of alfaxalone and xylazine achieves a longer duration of surgical anesthesia.[29] Another study found that survival rates were unacceptable when alfaxalone was administered IP before abdominal surgery, with a better survival rate after SQ administration.[2] Other studies also demonstrated survival rates were better with the combination of alfaxalone,

Submitted: 08 Sep 2023. Revision requested: 17 Oct 2023. Accepted: 06 Dec 2023.
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medetomidine, and butorphanol after SQ as compared with IP administration.[14,31]

We hypothesized that SC injection of an alfaxalone-xylazine-buprenorphine (AXB) combination would provide rapid and smooth induction of a surgical plane of anesthesia in a variety of mouse strains and that AXB anesthesia would be associated with fewer cardiac side effects during an echocardiogram as compared with KX in Crl:CFW(SW) mice.

Materials and Methods

Animals and housing facility. The study was comprised of Crl:*CFW*(SW) (CFW) and C57Bl/6NCrl (C57Bl/6) mice purchased from Charles River Laboratories (Wilmington, MA) and NCr-Foxn1^{nu} (NU/NU) and NOD.Cg-Prkdc^{SCID}Il2rg^{tm1Wjl}/SzJCr (NSG) mice obtained from an in-house breeding colony. All mice were housed at the National Cancer Institute (Frederick, MD), an AAALAC internationally accredited facility that follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the *Guide for Care and Use of Laboratory Animals*[24] All procedures were approved by the IACUC of the National Cancer Institute (Frederick, MD).

All mice used in this study were between 8 to 12 wk of age. Mice were housed in individually ventilated cages at 68 to 72 °F and 30 to 70% humidity on a 12:12-h light:dark cycle. CFW and C57Bl/6 mice were housed in nonautoclaved cages that were handled in an Allentown Phantom Animal Transfer Station); NU/NU and NSG mice were housed in autoclaved cage and handled in a biosafety cabinet (Labgard Class II/B2, model no. NU-5435-600, Nuaire, Plymouth, MN).

Both direct colony and dirty bedding sentinel testing were used to ensure mice were free of ectoparasites (ticks, fleas, lice, mites, and *Encephalitozoon cuniculi*), endoparasites (tapeworms, pinworms, helminths, *Spironucleus muris*, and *Giardia*), mouse adenovirus, mouse thymic virus, mouse parvovirus, pneumonia virus of mice, Sendai virus, Reovirus 3, rotavirus/epizootic diarrhea of infant mice, mouse polyoma virus, K virus, murine cytomegalovirus, lymphocytic choriomeningitis virus, lactate dehydrogenase elevating virus, murine hepatitis virus, ectromelia virus, minute virus of mice, Theiler encephalomyelitis virus, Hantaviruses, *Streptobacillus moniliformis*, *Corynebacterium kutscheri*, *Citrobacter rodentium*, *Salmonella* spp., *Clostridium piliforme*, *Filobacterium rodentium*, *Streptococcus pneumoniae*, *Mycoplasma pulmonis*, and *Helicobacter* spp. murine norovirus, *Corynebacterium bovis*, and murine chapparvovirus are not excluded. The suite used for immunocompromised mice also excludes *Demodex muris*, Trichomonads, Entamoeba, murine norovirus, murine chapparvovirus, *Corynebacterium bovis*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Staphylococcus aureus*, *Pneumocystis carinii*, *Pseudomonas aeruginosa*, *Bordetella bronchiseptica*, *Mycoplasma arthritidis*, and *Rodentibacter pneumotropicus*.

Mice obtained from vendors were acclimated for one week after arrival. Food, bedding, and enrichment were the same in both standard and barrier-housed mice except that the water was acidified for mice used in the echocardiography experiment. All mice were fed ad libitum autoclaved pelleted feed (Rodent Diet 6% fat, autoclavable no. 1021; PMI, Test Diet, Purina) and provided with reverse osmosis purified water. Mice received acidified (pH 2.5 to 3.0) water while housed in the imaging facility during the echocardiography experiment. All mice were provided with cotton squares (NES3600, Ancare, Bellmore, NY) except for NU/NU mice, which received Enviro-dri (Shepherd Specialty Papers).

Dose-finding experiment. An initial study was performed to determine an appropriate dose of alfaxalone (10 mg/mL, Alfaxan Multidose, Jurox, KS City, MO) that would induce a surgical plane of anesthesia in combination with xylazine and buprenorphine in CFW mice. For this study, mice (15 female and 15 male CFW mice; n = 5 males and 5 females per dosing group) were assigned by cage to one of 3 alfaxalone doses administered in combination with 10 mg/kg xylazine (20 mg/mL, AnaSed, Akorn, Lake Forest, IL) and 0.1 mg/kg buprenorphine HCl (0.3 mg/mL, PAR Pharmaceutical, Chestnut, Ridge, NY); these doses were 40 mg/kg alfaxalone (40AXB), 45 mg/kg alfaxalone (45AXB), and 50 mg/kg alfaxalone (50AXB). Drugs were diluted with sterile 0.9% NaCl, combined into one syringe, and administered SC between the scapulae using a 25-gauge, 5/8-in. needle (BD, Franklin Lakes, NJ). Once mice became immobile, they were treated with artificial tear ointment (Lubricant PM Ointment, AACE, Morgantown, WV) and were maintained on a heating pad (37 °C; Thermo-Farm Animal Mat, K and H Pet Products, Colorado Springs, CO).

Time to loss of 3 reflexes was evaluated every 2 min to determine surgical plane of anesthesia: loss of righting reflex (LORR), tail pinch, and toe pinch.[8] Once a surgical plane was reached, tail and toe pinch reflexes were evaluated every 5 min. LORR was assessed by placing the mouse in dorsal recumbency until it was unable to return completely to sternal recumbency. Tail pinch reflex was evaluated by pinching the tail on 3 locations with fingertips: the base, middle, and tip of the tail. Toe pinch reflex was evaluated by pinching firmly with fingertips on both hind paws. All tail pinch and toe pinch reflex tests were performed by the same person. Immobilization was defined as LORR and the absence of voluntary movement. A surgical plane of anesthesia was defined to begin when a mouse lost all 3 reflexes in addition to the absence of spontaneous movement. Duration of the surgical plane of anesthesia was defined as the time between reaching a surgical plane and the time to recover the toe pinch reflex. Time to recover from anesthesia was defined as the interval between administering the injection and recovery of all 3 reflexes and spontaneous movement (Figure 1).

Respiratory rate was measured every 2 min until a surgical plane of anesthesia was reached by manually counting thoracic excursions during a 15-s interval. Respiratory rate, toe pinch, and tail pinch reflexes were continuously monitored every 5 min during the surgical plane of anesthesia. All observers were blind to the syringe contents during these evaluations.

The times to recovery of toe pinch, tail pinch, and righting reflex were recorded manually. When the mouse's righting reflex returned, they were returned to the home cage and provided diet gel (DietGel Recovery, Clear H2O, Westbrook, ME).

Use of alfaxalone combination in a surgical model. The goal of this experiment was to determine if the dose of the alfaxalone-based anesthetic combination identified above (AXB) would provide a depth of anesthesia sufficient for routine laparotomy. This study was performed using CFW mice (females, n = 5; males, n = 5).

Mice were anesthetized SC with 50AXB as described above. The times of injection, LORR, and loss of toe pinch reflex were recorded. Respiratory rate was monitored every 2 min until the surgical plane was achieved. Then respiratory rate, LORR, and loss of toe pinch reflex were monitored every 5 min until the end of the surgical procedure. Upon reaching a surgical plane of anesthesia, mice were moved over to a heating pad, and the eyes were lubricated with sterile artificial tear ointment. The abdomen was shaved and aseptically prepared with 3 alternating applications of iodine and isopropyl alcohol.

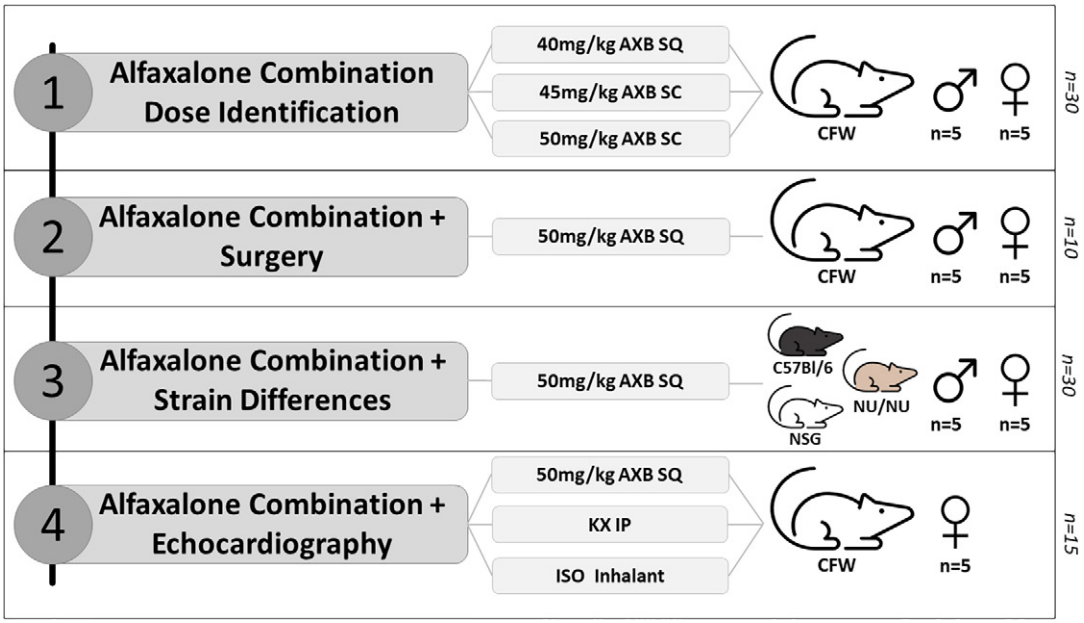

Figure 1. Flow chart to describe (1) dose-finding experiment using AXB, (2) surgery experiment using AXB, (3) strain difference comparison using AXB, and (4) echocardiography experiment comparing AXB, KX, and ISO.

A surgical drape (clear plastic, 8 in. × 8 in., Steris, Saxonburg, PA) was then placed over the mouse. The exploratory laparotomy included making a ventral midline abdominal incision through the skin with a scalpel blade (no. 15 Protected Disposable Scalpels, Bard-Parker, Danbury, CT) and extending the incision to 1.5 cm with a Metzenbaum scissor. Another incision was made into the linea alba to open the abdominal cavity, and the incision was extended to 1.5 cm with a Metzenbaum scissor. The abdominal contents were manipulated by moving the contents to the right and then to the left, exteriorizing the spleen and small intestines, and then returning them to the abdomen. The linea alba was closed with Monocryl 4-0 (Reverse Cutting, 19 mm, 3/8 c; Ethicon, Raritan, NJ) using a simple continuous pattern followed by closure of the skin layer with Autoclips (BD Autoclip, 9 mm; Thomas Scientific, Swedesboro, NJ). All mice received 0.05 mL of diluted 0.25% ropivacaine (0.5% ropivacaine HCl, Akorn, Lake Forest, IL) as a local analgesic after the abdominal wall was closed, before skin closure (Figure 1).

Total surgery time was between 5 to 10 min. Time to recovery of toe pinch reflex, tail pinch reflex, and righting reflex were recorded. Mice were moved into a cage with soft bedding (Diamond Soft Bedding Teklad 7089, Envigo, Indianapolis, IN) after all 3 reflexes returned to baseline, and return of spontaneous movement was confirmed. To assist in recovery,[12] a diet gel cup (DietGel Recovery, Clear H2O, Westbrook, ME) and meloxicam tablets (Mouse MD's Meloxicam, 0.125-mg tablet, bacon flavor, Bio-Serv, San Diego, CA) were provided for 3 d. To allow the mice to become familiar with the meloxicam tablet, a placebo tablet (Rodent MD's Placebo, 5-g tablet, grain-based bacon flavor, Bio-Serv, San Diego, CA) was offered on the day before surgery.

Mice were monitored daily for 7 d after surgery, at which time the incision sites were fully healed. Mice were then euthanized with CO_2 per IACUC guidelines. A necropsy was performed due to unexpected death, but we did not perform a necropsy on all mice that were euthanized.

Efficacy of alfaxalone combination in different strains. Five males and 5 females of C57Bl/6, NU/NU, and NSG mice ($n = 30$) were used for this study. 50AXB was administered as described above. LORR, tail pinch reflex, and toe pinch reflex were assessed in the same manner as above. Once mice lost righting reflex and spontaneous movement, they were moved over to a heating pad and administered eye ointment. The respiratory rate, tail pinch reflex, and toe pinch reflex were monitored every 5 min. Time to recovery of the reflexes and return of spontaneous movement was recorded. Mice were returned to their home cage and given a diet gel cup when their reflexes and voluntary movement returned (Figure 1).

Echocardiography. Fifteen female CFW mice were used in this study. Each mouse was assigned by cage to an anesthetic group: 1) 50AXB ($n = 5$); 2) 100 mg/kg ketamine (100 mg/mL ketamine hydrochloride, Covetrus, Portland, ME) and 10 mg/kg xylazine IP ($n = 5$); or 3) isoflurane (Covetrus, Portland, ME) ($n = 5$). Isoflurane (ISO) was administered with a vaporizer (Somni Scientific, South Park Township, PA) with filtered (22 µm) air as the carrier gas and a 1.5 L/min flow rate. Mice were induced with isoflurane in an induction chamber at 3% and maintained during imaging at 2% isoflurane with a nose cone. Upon confirmation of LORR and absence of spontaneous movement, the parasternum was shaved and a depilatory cream (Surgicream, Kanar, Carlstadt, NJ) was applied to reduce hair/transducer image artifacts. The depilatory cream was wiped off with acetic acid to counteract the pH of the Surgicream after 2 min. Mice were then placed on a heated (37 °C) imaging platform with limbs taped to the cardiac electrodes, and the eyes were lubricated. Echocardiography was performed 15 to 20 min after anesthetic induction using the VEVO 2100 Ultrasound Scanner (VisualSonics, Toronto, Canada) with a 30-MHz transducer (MS 400 VisualSonics), 449 frame rate, and 50-µm axial image resolution (Figure 1).

Warm (38.8 °C) ultrasound gel (Ultrasound Transmission Gel, AquasonicGel, Clinton Township, MI) was applied to the mouse to provide a consistent acoustic impedance between the transducer and mouse. Echocardiography was performed via manufacturer protocol: M mode, fractional shortening (FS), ejection fraction (EF), cardiac output (CO), end-systolic volume (V;s), and end-diastolic volume (V;d), and heart rate (HR)

were recorded and calculated over an interval of 8 heartbeats per mouse.

Statistical analysis. Pairwise group comparisons (R Statistical Software, v4.1.1, R Core Team, 2021) were made using a 2-sided unpaired Wilcoxon rank test for the dose-finding, surgical model, and strain comparison experiments. Mice that did not reach a surgical plane of anesthesia in the dose-finding experiment were assigned the maximum time recorded for the other mice (40 min + 1). This method of analysis considered that these mice responded less strongly than mice that did lose the toe pinch reflex.

Nonparametric rank-based statistics were used for data that did not have a normal (Gaussian) distribution.[21,24] The nonparametric 2-sided unpaired Wilcoxon rank test was used to analyze time to loss of pedal reflex in the dose-finding and strain comparison studies because some mice did not reach a surgical plane of anesthesia.

Echocardiogram data were normally distributed and did not require missing value imputation. The results for HR, CO, EF, FS, V;s, and V;d were measured and reported with an average ± SD over 8 heartbeats. Student *t* test was used to compare HR between groups.

In all cases, statistical significance was set at $P \leq 0.05$. Data points are reported as the average ± SD. Pilot data and previous studies[5,14,29,31] published on alfaxalone anesthesia in mice demonstrated relatively large effect sizes for the parameters measured in this study. Therefore, similar to previous studies,[5,14,29,31] group size was set to 5 mice to attain sufficient power.

Results

Dose-finding experiment. Three SC doses of alfaxalone were tested: 40, 45, and 50 mg/kg. In the 40AXB group, LORR and loss of tail pinch reflex occurred within 3 min after injection, but only 3 of the 5 female mice achieved a surgical plane of anesthesia. Among those that reached a surgical plane, its duration was 42.0 ± 7.0 min and time to recover from anesthesia in all 5 females was 54.8 ± 2.9 min. All 5 male mice achieved the surgical plane of anesthesia at this dose, although one mouse took 40 min to achieve a surgical plane of anesthesia and 2 mice had muscle tremors. Duration of surgical plane of anesthesia and time to recover from anesthesia were 33.4 ± 11.6 min and 71.6 ± 10.4 min, respectively, in males. In the 45AXB group, only 4 out of 5 female mice achieved a surgical plane of anesthesia whereas all 5 males achieved a surgical plane, with one male requiring 35 min. The total duration of surgical anesthesia in this group was 56.3 ± 2.5 min in females and 49 ± 11.8 min in males. The time to recover from anesthesia in this group was 79.4 ± 13.1 min in females and 83.6 ± 8.1 min in males. Four of the mice in the 40AXB and 45AXB groups continued to have involuntary muscle movement after achieving the surgical plane of anesthesia. In the 50AXB group, all mice achieved surgical plane of anesthesia within 7.7 ± 2.5 min in females and 12 ± 5.2 min in males. The total duration of the surgical plane of anesthesia was 48.6 ± 4.7 min in females and 60.8 ± 9.6 min in males. The time to recover from anesthesia in this group was 83.2 ± 17.8 min in females and 78.6 ± 7.6 min in males (Table 1).

Among the 3 doses tested, 50AXB most reliably induced a surgical plane of anesthesia in both sexes of CFW mice. In the 50AXB group, the duration of the surgical plane of anesthesia ($P = 1$, no difference, Figure 2A) and the time to recover from anesthesia ($P = 0.834$, no difference, Figure 2B) were not significantly different between males and females.

Use of alfaxalone combination in a surgical model (laparotomy). The 50AXB anesthetic combination provided sufficient depth and length of anesthesia to perform laparotomy in both female and male CFW mice (Table 2). All 10 mice (5 females and 5 males) survived the laparotomy procedure and recovered uneventfully with no complications. Mice were monitored every day for 7 d. The incision was fully healed on day 7 after surgery.

Efficacy of alfaxalone combination in different strains. The 50AXB combination produced a surgical plane of anesthesia in a majority of C57Bl/6, NU/NU, and NSG mice. Times to achieve surgical plane of anesthesia for each strain were as follows for females and males, respectively: C57Bl/6, 8.75 ± 11.2 and 7.8 ± 2.0 min; NU/NU, 13.4 ± 10.8 and 8.8 ± 2.8 min; and NSG, 12.8 ± 4.6 and 8.6 ± 3.6 min. There was no significant difference between the sexes within each strain in the time to reach a surgical plane of anesthesia. ($P > 0.05$). One female C57Bl/6 and one female NSG did not achieve a surgical plane of anesthesia (Table 2).

The durations of the surgical plane for females and males, respectively, of each strain were as follows: C57BL/6, 42.3 ± 11.2 and 51.6 ± 12.3 min; NU/NU, 76.8 ± 32.5 and 80.0 ± 1.2 min; and NSG, 56.0 ± 37.2 and 61.2 ± 10.2 min, with no significant difference between the sexes within each strain ($P > 0.05$) (Table 2).

Times to recover from anesthesia in females and males, respectively, in each strain were as follows: C57BL/6, 60.0 ± 13.7 and 75.6 ± 6.1 min; NU/NU, 120.4 ± 27.5 and 129.0 ± 17.0 min; and NSG, 106.6 ± 13.8 and 113.6 ± 10.5 min, with no significant differences between sexes for any strain ($P > 0.05$) (Table 2).

Echocardiography. Table 3 summarizes the echocardiography data from the anesthetic groups 50AXB, KX, and ISO in male and female CFW mice. CO (mL/min), FS (%), EF (%), HR (bpm), V;s (μL), and V;d (μL) were calculated using the M mode from the short axis view (Figure 3). One mouse in the KX

Table 1. Response to different doses of subcutaneous injection of AXB

Dosage group (mg/kg)	Sex (No.)	No. (%) that achieved sx plane	Duration of sx plane of anesthesia (min)	Time to recover from anesthesia (min)	No. that had involuntary muscle movement after achieving LORR
40AXB	F (n = 5)	3 (60%)	42.0 ± 7.0	54.8 ± 2.9	1
	M (n = 5)	5 (100%)	33.4 ± 11.6	71.6 ± 10.4	2
45AXB	F (n = 5)	4 (80%)	56.3 ± 2.5	79.4 ± 13.1	0
	M (n = 5)	5 (100%)	49.0 ± 11.8	83.6 ± 8	1
50AXB	F (n = 5)	5 (100%)	48.6 ± 4.7	83.2 ± 17.8	0
	M (n = 5)	5 (100%)	60.8 ± 9.6	78.6 ± 7.6	0

Summary of the number of mice reaching a surgical place of anesthesia, duration of the surgical plane of anesthesia, time to recover from anesthesia, and number of mice with involuntary muscle movement after loss of righting reflex. Data are shown for all 3 doses in both sexes of CFW mice. Data are given as mean ± SD. LORR, loss of righting reflex; Sx plane, surgical plane of anesthesia.

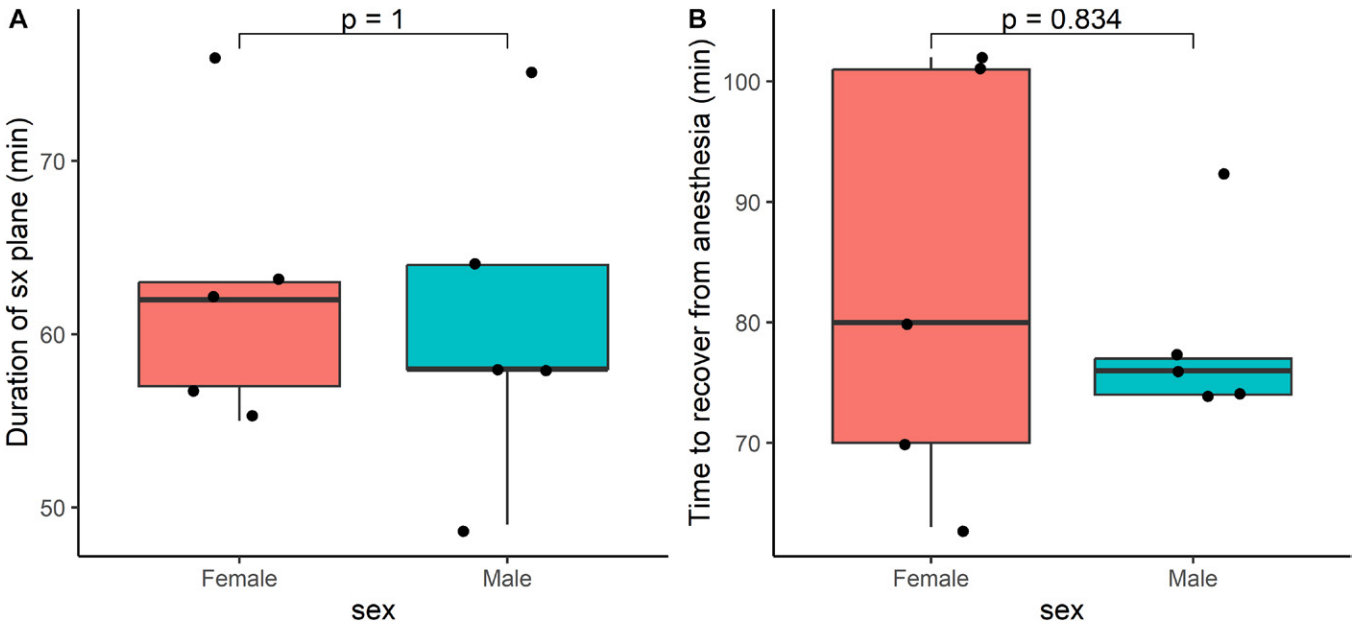

Figure 2. Comparison of 50AXB in female and male CFW mice. (A) Sex comparison for durations of the surgical plane of anesthesia and (B) time to recover from anesthesia. Data are given as mean ± SD. Each point represents an individual mouse. The box indicates the first and third quartile range with the whiskers extending to values within 1.5× the interquartile range of the dataset.

group died immediately after injection before echocardiography was performed and was excluded from statistical analysis and replaced by another mouse. All other mice recovered from anesthesia without complication. In all mice, V;s, EF, and FS were within normal physiologic limits, with no significant difference between the anesthetic groups. Heart rates were significantly higher in the 50AXB group as compared with the KX group (P = 0.001, Figure 4). However, 50AXB still induced significant bradycardia as compared with ISO (P < 0.0002, Figure 4). The CO and V;d were not significantly different between 50AXB and ISO groups, but the KX group had significantly lower CO and V;d as compared with ISO (P = 0.0044 and P = 0.038, respectively, Table 3).

Discussion

Identifying appropriate injectable anesthetic combinations facilitates the study of surgical models of ischemic cardiomyopathy, cardiac hypertrophy, and heart failure in mice. A suitable combination must, at minimum, safely induce a surgical plane of anesthesia for 30 min with minimal impact on the cardiovascular

Table 2. Response to 50AXB in CFW, C57Bl/6, NU/NU, and NSG mice

Strain	Sex (no.)	No. (%) that achieved sx plane	Time to sx plane (min)	Duration of sx plane (min)	Time to recover from anesthesia (min)
CFW	F (n = 5)	5 (100%)	12.0 ± 6.4	48.6 ± 4.7	83.2 ± 17.8
	M (n = 5)	5 (100%)	7.4 ± 3.0	60.8 ± 9.6	78.6 ± 7.6
C57Bl/6	F (n = 5)	4 (80%)	8.75 ± 11.2	42.3 ± 11.2	60.0 ± 13.7
	M (n = 5)	5 (100%)	7.8 ± 2.0	51.6 ± 12.3	75.6 ± 6.1
NU/NU	F (n = 5)	5 (100%)	13.4 ± 10.8	76.8 ± 32.5	120.4 ± 27.5
	M (n = 5)	5 (100%)	8.8 ± 2.8	80.0 ± 1.2	129.0 ± 17.0
NSG	F (n = 5)	4 (80%)	12.8 ± 4.6	56.0 ± 37.2	106.6 ± 13.8
	M (n = 5)	5 (100%)	8.6 ± 3.6	61.2 ± 10.2	113.6 ± 10.5

Summary of time to achieve a surgical plane of anesthesia, duration of the surgical plane, and time to recover from anesthesia in 4 strains of mice. Data from CFW mice listed were collected in the dose-finding experiment. Data are given as mean ± SD. Sx plane, surgical plane.

Table 3. Summary of numerical cardiac values calculated by M mode

Anesthesia group (no.)	V;s (µL)	V;d (µL)	EF (%)	FS (%)	CO (mL/min)	HR (bpm)
AXB (n = 5)	22.8 ± 5.7	64.3 ± 15.2	64.6 ± 1.2	34.7 ± 0.9	12.4 ± 3.6	295.4 ± 29[a]
KX (n = 5)	25.8 ± 2.0	73.9 ± 4.2[†]	64.7 ± 4.3	35.1 ± 3.3	9.2 ± 2.2[b]	185.8 ± 38.9[a]
ISO (n = 5)	22.4 ± 14.1	60.8 ± 9.8[†]	65.1 ± 15.9	36.2 ± 11.5	17.6 ± 3.8[b]	454 ± 41.7[a]

Data are given as mean ± SD for the 6 measured cardiac parameters: V;s, end-systolic volume; V;d, end-diastolic volume; EF, ejection fraction; FS, fractional shortening; CO, cardiac output; HR, heart rate.
[a]Significant difference in all groups.
[b]Significant difference in KX and ISO groups.

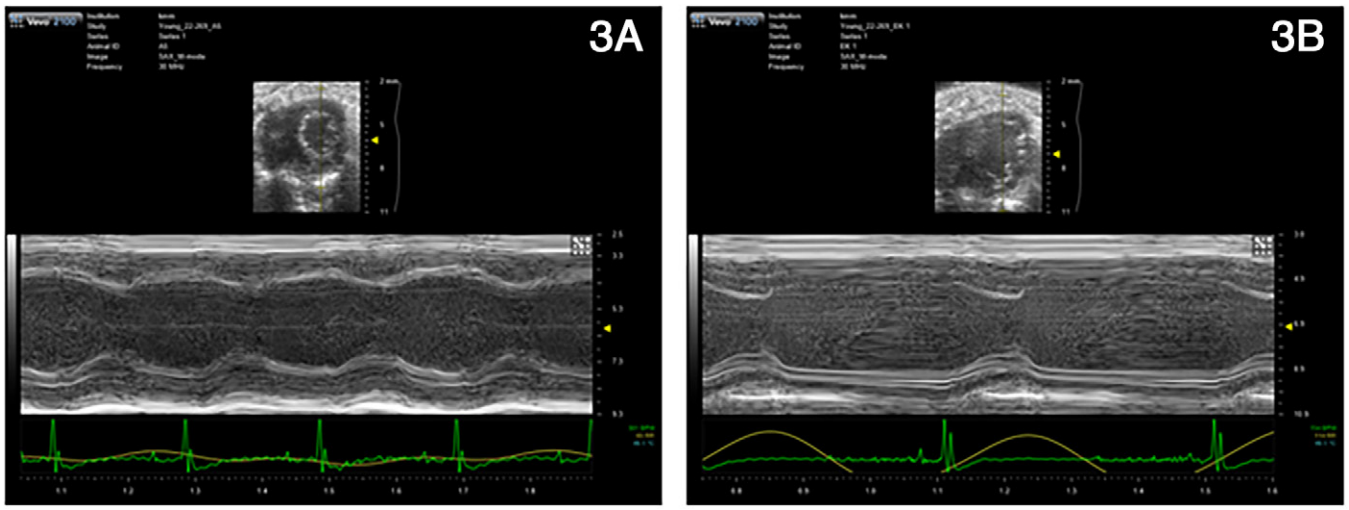

Figure 3. (A) Short-axis view (top) M mode captured based on the short-axis view imaged (bottom) of mouse anesthetized with AXB captured over 2 s. (B) Short-axis view (top) M mode captured (bottom) of mouse anesthetized with KX captured over 1.6 s.

function. Ideally, the same anesthetic combination could be used in multiple strains and sexes.

Alfaxalone has gained popularity because of its ability to provide reliable anesthesia with little to no cardiovascular side effects.[5,14,18,19,29] Previous studies have indicated that alfaxalone anesthetic combinations induce reliable anesthesia in several strains of mice at doses ranging from 60 to 120 mg/kg, but mice did not reach a surgical plane of anesthesia at lower doses.[5,14,29] In dogs and cats, alfaxalone anesthesia can lead to hypoventilation, respiratory apnea, and cardiorespiratory depression.[22,23] A multimodal anesthetic combination should allow the use of a lower dose of alfaxalone and thereby limit the possibility of these side effects. In previous studies, α_2-agonists and opioids have been used for this purpose, including combinations with alfaxalone-medetomidine-butorphanol and alfaxalone-xylazine.[5,31] We chose to use xylazine rather than medetomidine because medetomidine is 10 times more potent and, as an α_2-adrenoceptor agonist, will cause greater vasoconstriction, which could lead to bradycardia and hypotension.[8] Because a potent opioid can further lower the required dose of anesthetic dose, we opted to use buprenorphine instead of butorphanol in this study. Buprenorphine is a partial μ-receptor agonist and κ-receptor antagonist, whereas butorphanol is a μ-receptor antagonist and κ-receptor agonist.[8,10] In a pilot to the current study, we used an alfaxalone-midazolam combination in 3 female mice of unknown strain and age. Three groups (with one mouse in each group) were administered alfaxalone (40, 60, or 80 mg/kg) + midazolam (5 mg/kg) SC. In all the groups, the mice experienced significantly prolonged sedation, poor recovery, and respiratory arrest. Therefore, we did not investigate this combination further. The poor results of the combination possibly occurred because midazolam and alfaxalone act at the same GABA receptor,[8] thus potentiating the sedative effect.

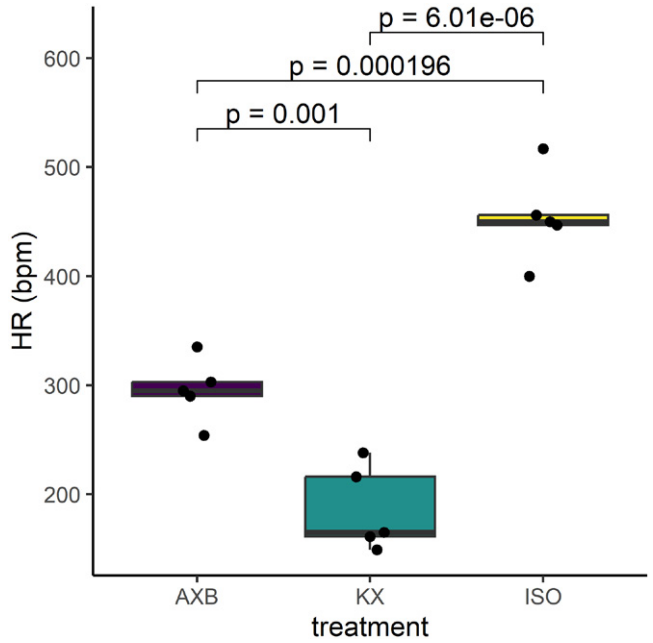

Figure 4. Heart rate in mice anesthetized with 50AXB-, KX-, and ISO-treated groups. Each point represents an individual mouse. The box indicates the first and third quartile range with the whiskers extending to values within 1.5× the interquartile range of the dataset.

Our data showed that the 50AXB anesthetic combination, given SC, provided appropriate anesthesia for an exploratory laparotomy. As reported previously, IP administration of an alfaxalone-xylazine combination resulted in an unacceptably high mortality rate in mice that underwent laparotomy.[5]

In our pilot study of the IP administration route, CFW mice (2 males and 2 females per group) given alfaxalone (40 or 60 mg/kg) + xylazine (10 mg/kg) + buprenorphine (0.1 mg/kg) did not reach a surgical plane of anesthesia. Only 2 males and one female in the 80-mg/kg group achieved a surgical plane of anesthesia. Moreover, their surgical planes lasted over 3 h. At this point, we elected not to pursue further study using IP administration of AXB.

Previous investigations[5,14,29] used higher doses of alfaxalone to achieve a surgical plane of anesthesia, but our mice reached a surgical plane at only 50 mg/kg, perhaps due to our use of both buprenorphine and a SC route. This dose was effective for an exploratory laparotomy. Mice remained at a surgical plane of anesthesia for the duration of the surgery and recovered without complication. If a mouse did not reach a surgical plane of anesthesia, a supplemental dose of alfaxalone (10 to 20 mg/kg) could be provided. However, this was unnecessary in our study.

While this worked equally for both sexes within each strain/stock, there were differences in the duration of the surgical plane and recovery time between strains.

CD1, C57BL/6, BALB/c, and ICR mouse strains were used in previous studies of alfaxalone.[5,31] After determining an appropriate dose and route of 50AXB in CFW mice, we tested other strains commonly used at our institution (C57Bl/6, NU/NU, and NSG mice). While this worked equally for both sexes within each strain/stock, there were differences in the duration of the surgical plane and recovery time between strains, consistent with other studies.[5,31] The longer duration of anesthesia in these immunocompromised strains could be shortened by the use of a reversal agent, atipamezole (α_2-antagonist), or possibly further lowering the dose of alfaxalone. Although strain differences are present, the 50AXB combination did meet our requirement for a minimum 30-min duration of the surgical plane of anesthesia in all stocks/strains.

Like all anesthetic protocols, the 50AXB combination entails risks. Two C57BL/6 males died after SQ administration of alfaxalone; both of these mice had a dermal injury that was visible at necropsy, was not in the same location as the injection, and was likely caused by interanimal aggression. These 2 mice were excluded from the data because the injuries may have affected alfaxalone absorption. The mechanism underlying these deaths could be related to that of the deaths that occurred after IP alfaxalone injection and laparotomy.[5]

Several previous studies[5,7,29,34] reported greater sensitivity to alfaxalone in female mice as compared with males, perhaps due to a biological difference in sensitivity to certain hormones. A previous pharmacokinetic study of alfaxalone noted that because alfaxalone is a derivative of progesterone, its absorption and metabolism may be affected by endogenous steroids.[34] We did not find any difference in the duration of the surgical plane between sexes in CFW, C57BL/6, NU/NU, or NSG mice. This may be due to the lower dose and SC route of alfaxalone used in our study.

KX is known to cause significant bradycardia, and vasoconstriction and is not recommended for experimental studies that involve the heart.[8,11,20,25,26,30,32,36] A reported benefit of alfaxalone is that it has minimal side effects on the cardiovascular system when given at a clinical dose.[5,13,14,18,19] To determine if AXB might be superior to KX, we performed echocardiograms in mice anesthetized with these combinations to evaluate cardiac function. Isoflurane was used as a control anesthetic because it has minimal cardiovascular effects.[11,20,26,30,32] Our data on HR were consistent with findings from previous studies showing that KX anesthesia causes significant bradycardia.[11,20,26,30,32,36] We found no significant differences in other cardiac measurements between AXB and KX groups. When comparing ISO and KX groups, the KX group had significantly lower diastolic volume, CO, and HR[11,30,32,35] (Table 3). The AXB group in comparison to ISO had a significantly lower HR with no statistical differences between other measured echocardiographic parameters. KX was a less reliable anesthetic combination overall. One mouse in the KX group died shortly after injection before an echocardiography could be performed and had to be replaced with another female. Mice in the KX group lost their righting reflex and tail pinch reflex; however, none reached a surgical plane of anesthesia at the dose used. Surgery may require a higher dose of KX, which would also exacerbate the bradycardia. Our data indicated that among the 3 methods we studied, ISO would be the best choice for cardiovascular studies. However, if an inhalant anesthetic is not feasible, injectable AXB may be superior to KX for cardiac models. Our echocardiography study used only one strain, CFW, and one sex, female. Responses to specific anesthetic regimens should be evaluated in strains of interest before being used for experimental purposes.

Overall, our findings suggest that SC administration of 50 mg/kg alfaxalone-10 mg/kg xylazine-0.1 mg/kg buprenorphine HCl is a viable anesthetic option for inducing a surgical plane of anesthesia in CFW, C57Bl/6, NU/NU, and NSG mice, regardless of sex. In addition, 50AXB may be superior to KX when there is a concern for adverse effects in mouse cardiac models.

Acknowledgments

This project was funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261201500003I. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. The authors thank Drs. Duncan Donohue and Tyler Malys (Statistical Consulting and Scientific Programming Group, Data Management Services) for statistical analysis of the data. Dr. Melinda Hollingshead (Developmental Therapeutics Program, National Cancer Institute) provided animals (NU/NU and NSG) for the study. Thanks to Dr. Julie Holdridge, DACLAM, for collecting the pilot data that led to this study.

References

1. **Adami C, d'Ovidio D, Casoni D.** 2016. Alfaxalone versus alfaxalone-dexmedetomidine anaesthesia by immersion in oriental fire-bellied toads (Bombina orientalis). Vet Anaesth Analg **43**:326–332.
2. **Albrecht M, Henke J, Tacke S, Markert M, Guth B.** 2014. Influence of repeated anaesthesia on physiological parameters in male Wistar rats: a telemetric study about isoflurane, ketamine-xylazine and a combination of medetomidine, midazolam and fentanyl. BMC Vet Res **10**:310.
3. **Buitrago S, Martin TE, Tetens-Woodring J, Belicha-Villanueva A, Wilding GE.** 2008. Safety and efficacy of various combinations of injectable anesthetics in BALB/c mice. J Am Assoc Lab Anim Sci **47**:11–17.
4. **Chu D, Jordan M, Kim J, Couto M, Roos K.** 2006. Comparing isoflurane with tribromoethanol anesthesia for echocardiographic phenotyping of transgenic mice. J Am Assoc Lab Anim Sci **45**:8–13.
5. **Erickson RL, Blevins CE, Souza Dyer C, Marx JO.** 2019. Alfaxalone-xylazine anesthesia in laboratory mice (*Mus musculus*). J Am Assoc Lab Anim Sci **58**:30–39.
6. **Eshar D, Beaufrere H.** 2020. Anesthestic effects of alfaxalone-ketamine, alfaxalone-ketamine-dexmedetomidine, and alfaxalone-butorphanol-midazolam administered intramuscularly in five-striped palm squirrels (*Funambulus pennantii*). J Am Assoc Lab Anim Sci **59**:384–392.
7. **Fink G, Sarkar DK, Dow RC, Dick H, Borthwick N, Malnick S, Twine M.** 1982. Sex difference in response to alphaxalone anaesthesia may be oestrogen-dependent. Nature **298**:270–272. 10.1038/298270a0.
8. **Fish R, Danneman P, Brown M, Karas A, editors.** 2008. Anesthesia and analgesia in laboratory animals. Burlington (MA): Academic Press.
9. **Fox J, Anderson L, Otto G, Pritchett-Corning K, Whary M, editors.** 2015. Laboratory animal medicine. Waltham (MA): Elsevier.
10. **Gades N, Danneman P, Wixson S, Tolley E.** 2000. The magnitude and duration of the analgesic effect of morphine, butorphanol, and buprenorphine in rats and mice. Contemp Top Lab Anim Sci **39**:8–13.
11. **Gao S, Ho D, Vatner D, Vatner S.** 2011. Echocardiography in mice. Curr Protoc Mouse Biol **1**:71–83.
12. **Gates K, Alamaw E, Jampachaisri K, Huss M, Pacharinsak C.** 2023. Efficacy of supplemental diet gels for preventing post operative weight loss in mice (*Mus musculus*). J Am Assoc Lab Anim Sci **62**:87–91.
13. **Heng K, Marx O, Jampachairsi J, Huss MK, Pacharinsak C.** 2020. Continuous rate infusion of alfaxalone during ketamine-xylazine anesthesia in rats. J Am Assoc Lab Anim Sci **59**:170–175.

14. **Higuchi S, Yamada R, Hashimoto A, Miyoshi K, Yamashita K, Ohsugi T.** 2016. Evaluation of a combination of alfaxalone with medetomidine and butorphanol for inducing surgical anesthesia in laboratory mice. Jpn J Vet Res **64**:131–139.
15. **Hohlbaum K, Bert B, Dietze S, Palme R, Fink H, Thöne-Reineke C.** 2018. Impact of repeated anesthesia with ketamine and xylazine on the well-being of C57BL/6JRj mice. PLoS One **13**:e0203559.
16. **Jaber S, Hankenson F, Heng K, McKinstry-Wu A, Kelz M, Marx J.** 2014. Dose regimens, variability, and complications associated with using repeat-bolus dosing to extend a surgical plane of anesthesia in laboratory mice. J Am Assoc Lab Anim Sci **53**:684–691.
17. **Kawahara Y, Tanonaka K, Daicho T, Nawa M, Oikawa R, Nasa Y, Takeo S.** 2005. Preferable anesthetic conditions for echocardiographic determination of murine cardiac function. J Pharmacol Sci **99**:95–104.
18. **Keates H, Whittem T.** 2012. Effect of intravenous dose escalation with alfaxalone and propofol on occurrence of apnoea in the dog. Res Vet Sci **93**:904–906.
19. **Lee J, Suh S, Choi R, Hyun C.** 2016. Cardiorespiratory and anesthetic effects produced by the combination of butorphanol, medetomidine, and alfaxalone administered intramuscularly in Beagle dogs. J Vet Med Sci **77**:1677–1680.
20. **Lindsey M, Kassiri Z, Virag J, de Castro Brás L, Scherrer-Crosbie M.** 2018. Guidelines for measuring cardiac physiology in mice. Am J Physiol Heart Circ Physiol **314**:H733–H752.
21. **Mishra P, Pandey CM, Singh U, Keshri A, Sabaretnam M.** 2019. Selection of appropriate statistical methods for data analysis. Ann Card Anaesth **22**:297–301.
22. **Muir W, Lerche P, Wiese A, Nelson L, Pasloske K, Whittem T.** 2008. Cardiorespiratory and anesthetic effects of clinical and supraclinical doses of alfaxalone in dogs. Vet Anaesth Analg **35**:451–462.
23. **Muir W, Lerche P, Wiese A, Nelson L, Pasloske K, Whittem T.** 2009. The cardiorespiratory and anesthetic effects of clinical and supraclinical doses of alfaxalone in cats. Vet Anaesth Analg **36**:42–54.
24. **Nahm F.** 2016. Nonparametric statistical tests for the continuous data: the basic concept and the practical use. Korean J Anesthesiol **69**:8–14.
25. **National Research Council.** 1996. Guide for the care and use of laboratory animals. Washington (DC): National Academy Press.
26. **Pistner A, Belmonte S, Coulthard T, Blaxall B.** Murine echocardiography and ultrasound imaging. JoVE. [Cited: 19 Nov 2022]. Available at: https://www.jove.com/v/2100/murine-echocardiography-and-ultrasound-imaging
27. **Roughan JV, Ojeda B, Flecknell PA.** 1999. The influence of pre-anaesthetic administration of buprenorphine on the anaesthetic effects of ketamine/medetomidine and pentobarbitone in rats and the consequences of repeated anaesthesia. Lab Anim **33**:234–242.
28. **Schuetze S, Manig A, Ribes S, Nau R.** 2019. Aged mice show an increased mortality after anesthesia with a standard dose of ketamine/xylazine. Lab Anim Res **35**:8.
29. **Siriarchavatana PD, Ayers J, Kendall LV.** 2016. Anesthetic activity of alfaxalone compared with ketamine in mice. J Am Assoc Lab Anim Sci **55**:426–430.
30. **Stypmann J, Engelen M, Epping CVM, van Rijen H, Milberg P, Bruch C, Breithardt G, Tiemann K, Eckardt L.** 2006. Age and gender related reference values for transthoracic Doppler-echocardiography in the anesthetized CD1 mouse. Int J Cardiovasc Imaging **22**:353–362.
31. **Tsukamoto Y, Yamada N, Miyoshi K, Yamashita K, Ohsugi T.** 2019. Anesthestic effect of a mixture of alfaxalone, medetomidine, and butorphanol for inducing surgical anethesia in ICR, BALB/c, and C57BL/6 mouse strains. J Vet Med Sci **81**:937–945.
32. **Vinhas M, Araújo AC, Ribeiro S, Rosário LB, Belo JA.** 2013. Transthoracic echocardiography reference values in juvenile and adult 129/Sv mice. Cardiovasc Ultrasound **11**:12.
33. **West S, Lee J, Johns T, Nunamaker E.** 2020. Intraperitoneal alfaxalone and alfaxalone-dexmedetomidine anesthesia in Sprague-Dawley Rats (*Rattus norvegicus*). J Am Assoc Lab Anim Sci **59**:531–538.
34. **White KL, Paine S, Harris J.** 2017. A clinical evaluation of the pharmacokinetics and pharmacodynamics of intravenous alfaxalone in cyclodextrin in male and female rats following a loading dose and constant rate infusion. Vet Anaesth Analg **44**:865–875.
35. **Yang X, Liu Y, Rhaleb N, Kurihara N, Kim HE, Carretero OA.** 1999. Echocardiographic assessment of cardiac function in conscious and anesthetized mice. Am J Physiol **277**:H1967–H1974.
36. **Zhang XX, Du ZD, Wen SG, Sun XP.** 2017. Echocardiographic evaluation of coronary abnormalities and cardiac function in a murine model of Kawasaki disease using high-frequency ultrasound. Chin Med J (Engl) **130**:1467–1474.